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In re application of: Suresh K. Arya, Ph.D.

Application No. 10/731,988

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For: LENTIVIRUS VECTOR SYSTEM

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DECLARATION UNDER 37 C.F.R. § 1.131

1. I, Suresh K. Arya, Ph.D, am the inventor named in the above-referenced patent application.
2. I have read and understand the above-referenced patent application, including the pending claims, and the Office action dated January 3, 2005.
3. It is my understanding that on page 6 of the Office action of January 3, 2005, pending claims 7-15, 17, 21, and 43-46, were rejected as allegedly unpatentable over Poeschla *et al.* (*J. Virol.* 72:6527-36, 1998) and MacCann *et al.* (*J. Virol.* 71:4133-7, 1997).
4. The Poeschla *et al.* article was published in August 1998. I was in possession of the claimed invention prior to August 1998. Accompanying this declaration as Exhibit A is a copy of my publication (Arya *et al.*, *Hum. Gene Ther.* 9:1371-80, 1998), which was published in June 1998. This article discloses HIV-2 packaging vectors, such as clone SD36, that contain deletion upstream and downstream of the splice donor site. Therefore, I invented the subject matter of the present application prior to publication of the Poeschla *et al.* article in August 1998.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Suresh K. Arya
Dr. Suresh K. Arya

6/27/05
Date

XP-000923199

HUMAN GENE THERAPY 9:1371-1380 (June 10, 1998)
Mary Ann Liebert, Inc.

10/06/1998
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Human Immunodeficiency Virus Type 2 Lentivirus Vectors for Gene Transfer: Expression and Potential for Helper Virus-Free Packaging

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ABSTRACT

In addition to the long-term expression of the transgene provided by all retroviral vectors, lentiviruses present the opportunity to transduce nondividing cells and potentially achieve regulated expression. The development of lentiviral vectors requires the design of transfer vectors to ferry the transgene with efficient encapsidation of the transgene RNA and with full expression capability, and of a packaging vector to provide packaging machinery *in trans* but without helper virus production. For both vectors, a knowledge of packaging signal is required—the signal to be included in the transfer vector but excluded from the packaging vector. Among the human lentiviruses, human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2), we think HIV-2 is better suited for gene transfer than HIV-1. It is less pathogenic and thus safer during design and production; its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes. In HIV-1 infection, it is less likely to recombine with the resident HIV-1, and it may itself downregulate HIV-1 expression. Evidently, elements located both upstream and downstream of the splice donor site in the leader sequence participated in RNA encapsidation and these sequences appeared necessary and sufficient. Deletion of both sequence elements resulted in a dramatic curtailment of RNA encapsidation and helper virus production. This was accompanied by some but acceptable loss of gene expression capability. The helper virus-free phenotype and expression capability of the double mutant was maintained upon replacement of its 3' long terminal repeat with a minigene cassette containing a transcriptional termination signal and a drug resistance marker gene. Deletion of the splice donor site itself had a dramatic negative effect on gene expression, supporting the important role of this element in the life of RNA.

OVERVIEW SUMMARY

The human immunodeficiency virus type 2 (HIV-2) lentivirus contains elements in its leader sequence located both upstream and downstream of the splice donor site that participate in RNA encapsidation. These elements appeared to be necessary and sufficient for packaging. Deletion of both upstream and downstream elements was clearly beneficial for curtailing helper virus protection, although this was accompanied by some but acceptable loss of expression capability. The helper virus-free phenotype of the packaging vector could be further ensured without additional loss of expression by replacing the 3' long terminal repeat with a minigene containing a heterologous transcriptional termination signal and a selection marker gene. Relevant to the

design of transfer vector, deletion of the splice donor site itself had a dramatic negative effect on viral gene expression.

INTRODUCTION

RETROVIRAL VECTORS continue to be the vehicles of choice for gene transfer requiring long-term expression (Anderson, 1992; Mulligan, 1993; Crystal, 1995; Arya and Gallo, 1996a). Because of their possession of specific regulatory genes, lentiviruses are unique among retroviruses in being able to infect quiescent cells. Their other regulatory genes modulate, positively or negatively, viral gene expression. Thus, these retroviruses can provide vectors to transfer genes into nondividing cells, including stem cells, with the possibility for reg-

ulated expression. Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2) are among the most studied lentiviruses, and a great deal is known about their gene structure and function. They are ideal candidates for vector development. Recent reports of the successful use of HIV-1-derived transfer vectors to transduce nondividing neuronal cells *in vivo* (Naldini *et al.*, 1996; Blomer *et al.*, 1997; Zufferey *et al.*, 1997) and CD34⁺ hematopoietic cells *in vitro* (Akkina *et al.*, 1996) have renewed and heightened the interest in lentiviral vectors. These studies generally used pseudovirions encasing HIV-1 vector into heterologous vesicular stomatitis virus (VSV) envelope glycoprotein G. However, the possible toxicity of VSV G protein for the packaging cell line and for *in vivo* administration, along with possible immune rejection, remains a concern. Efforts to create homologous packaging cell lines continue (Poeschla *et al.*, 1996; Corbeau *et al.*, 1996). A novel use of VSV as a targeted gene transfer vector was recently described (Schnell *et al.*, 1997). In this design, VSV genetic apparatus carries the transgene, but it is enclosed in a shell consisting of CD4 and CXCR4 receptor proteins.

A vector system consisting of lentiviral genetic elements requires (i) the design of a transfer vector that will shuttle the transgene with the potential for regulation and for high-titer encapsidation and (ii) creation of a packaging cell line that will encapsidate vector RNA but not the viral RNA encoding the packaging components and thus be helper virus free. Because packaging is governed by specific sequence elements, the opposing requirements—inclusion in the transfer vectors and exclusion from the packaging vectors—necessitate a detailed knowledge of the packaging signal. For HIV-1, previous studies have variously reported the location of this signal in the 5'-leader sequence downstream (Richardson *et al.*, 1993; Luban and Goff, 1994; Parolin *et al.*, 1994), upstream (Kim *et al.*, 1994), and downstream and upstream (McBride and Pangniban, 1996; McBride *et al.*, 1997) of the splice donor site. A previous suggestion regarding the participation of the *rev* response element (RRE) in HIV-1 RNA packaging (Richardson *et al.*, 1993) apparently now has been discounted (McBride *et al.*, 1997).

For HIV-2, we have previously reported that the leader sequence of this lentivirus contains a packaging signal downstream of the splice donor site but that this signal was not the sole determinant of RNA encapsidation (Garzino-Demo *et al.*, 1995). We noted that although a deletion of this downstream region caused packaging defect, it did not abolish encapsidation. To characterize the packaging signal further and, more importantly, to design packaging vectors that will express components needed for packaging but not encapsidate the coding RNA, that is, not produce helper virus, we studied the effect of additional leader sequence mutants as well as the effect of the replacement of the 3' long terminal repeat (LTR) on expression and packaging. While these studies were in progress, a report appeared that confirmed some of our previous results but suggested that the downstream sequence elements made only a minor or no contribution to RNA encapsidation and that the major element was located upstream of the splice donor site (McCann and Lever, 1997).

In this report, we confirm and extend our previous observations on encapsidation and note that both upstream and downstream elements participate in RNA encapsidation and that the

magnitude of the effect of the specific sequence elements may depend on the cell type. We also report that deletion of both the upstream and downstream elements dramatically curtails helper virus production, with some but acceptable loss of expression capability. The replacement of the 3' LTR with a cassette containing a heterologous transcriptional terminator and drug selection marker gene ensures the helper virus-free phenotype without further loss of the expression of the packaging components.

MATERIALS AND METHODS

Proviral DNA clones

Parental biologically active provirus molecular clone pROD-1 of HIV-2 (ROD) virus was first modified to obtain the clone termed pROD-3. This clone was obtained by inserting a synthetic linker with a multiple cloning site and a stop codon in the *nef* gene at a site 69 amino acids downstream of the *nef* initiator codon, thus truncating it and providing new cloning sites. The pROD-3 clone was phenotypically equivalent to the parental pROD-1 clone (Arya and Sadaie, 1993; Arya and Mohr, 1994). For introducing mutations in the leader sequence, a 5'-*EcoRI-EcoRI* (nucleotides 2,658) fragment of pROD-3 was subcloned into a plasmid vector. The *BglII* site (nucleotide 502) of this subclone was used to create endonuclease *Bal31* deletion mutants and an insertion of a synthetic *BssH2* site, thus providing subclones with deletions downstream of the splice donor site (nucleotide 470). The *BssH2* site was then used to create upstream deletion mutants employing synthetic linkers with additional *EagI* site at nucleotide 305. The viral fragment from the selected subclones was reinserted into an appropriately modified pROD-3 clone. For molecular clones containing puromycin resistance gene, the gene with or without a transcriptional termination [poly(A)] signal was inserted at the engineered multiple cloning site in the truncated *nef* gene of pROD-3 clone. All mutant clones were checked by DNA sequencing.

DNA transfection and antigen capture assays

For monolayers of epithelioid human embryonic kidney 293 or 293 T cells used interchangeably in these studies, $0.5\text{--}1.0 \times 10^6$ cells were transfected with 8–12 μg of proviral DNA by the calcium phosphate transfection protocol (Sadaie *et al.*, 1998; Arya, 1993), and cells and culture supernatants were harvested 3 days later. For suspension culture of human lymphoid CEM cells, $4\text{--}8 \times 10^6$ cells were transfected with 4–8 μg of proviral DNA by the DEAE-dextran protocol (Arya, 1990; Arya and Sethi, 1990), and cells and culture supernatants were harvested 5 days later. The CEM cultures were visually examined for syncytia formation before harvests. Viral particles in the supernatant were estimated by the standard antigen capture assay scoring for the p27 core protein (Arya and Gallo, 1996b; Al-Harthi *et al.*, 1998).

Infectivity assays

To determine the infectivity of the progeny virus, aliquots of cell-free culture supernatants from transfected cultures were

incubated with CEM cells for 2–4 hr, washed two times with phosphate-buffered saline (PBS) and once with complete culture medium, and incubated for 5 days. Cultures were visually examined for syncytia formation and supernatant harvested for analysis. Secondary virus production was evaluated by p27 core antigen capture assays.

RNA analysis

Cellular RNA was extracted by lysing cells with RNAzol (Tel-Test, Friendswood, TX) and RNA precipitated with isopropanol. The precipitate was dissolved, extracted with phenol-chloroform and re-ethanol precipitated. The precipitate was redissolved, treated with RNase-free DNase, extracted with phenol-chloroform, and ethanol precipitated. For viral particle-associated RNA, clarified culture supernatant was pelleted through a column of 20% glycerol in TNE (10 mM Tris-HCl pH 7.0, 0.15 M NaCl, 1 mM EDTA) by high-speed centrifugation (Beckman SW41 rotor at 33,000 rpm for 1 hr). The pellet was lysed with RNAzol and viral RNA extracted and DNase-treated as described above.

The abundance of viral RNA was estimated by slot-blot hybridization. Aliquots of cellular RNA (usually, 10–20 µg) or viral RNA (usually, half the initial amount) were denatured by heating RNA in 12× SSC, 12% formaldehyde at 65°C for 5 min followed by quick cooling. Denatured RNAs were further diluted with 15× SSC and two dilutions (1:1 and 1:5) were slot-blotted onto nitrocellulose membranes and hybridized with ³²P-labeled virus probe. Virus-specific RNA was quantitated by integrating the intensity of the bands with a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA) and intensity expressed in arbitrary units.

RESULTS

Two human cell lines, one epithelioid (293) and other lymphoid (CEM), were chosen for this study. The cell lines were transfected with the molecular clones and intracellular viral RNA and protein synthesis was measured for evaluating gene expression. To estimate helper virus production, extracellular release of particles containing viral RNA and proteins and their transmissibility was determined.

Human epithelioid 293 cells

Expression: Figure 1 shows the measurement of intracellular viral RNA and protein expression in cells transfected with the wild-type and mutant HIV-2(ROD) clones containing deletions in the 5' leader sequence. Expression of the mutant clone with a short deletion of 22 nucleotides (nucleotides 499–520) located downstream of the major splice donor site at nucleotide 470 and upstream of the gag ATG at nucleotide 546 (clone PK2) was not much different than the expression of the wild-type clone WT. No difference was observed either for viral RNA or viral protein synthesis, with the latter being measured by the estimation of the p27 core antigen in the cellular extracts. Extension of the deletion to 53 nucleotides (nucleotides 486–538) (Clone PK36) in this downstream region also did not affect the expression of vector RNA and proteins. Similar results were ob-

tained for the mutant clone with deletion (nucleotides 306–459) upstream of the splice donor site (clone SK36).

A deletion in the downstream region of HIV-2(ROD) similar in size to that of clone PK36 but extending into the splice donor site (clone PK8) had a detrimental effect on viral expression. Little or no viral RNA was detected in cells transfected with this clone. This is a clear and dramatic evidence for the importance of splicing in viral RNA processing and expression, and hence in its replication.

The clone containing deletions both downstream (nucleotides 482–538) and upstream (nucleotides 306–459) of the splice donor site in the leader sequence (clone SD36) displayed diminished RNA expression relative to the wild-type provirus (about one-half to one-third). This decrease was not paralleled exactly by the decline in intracellular core antigen accumulation, which may be related to the differences in the relative rates of synthesis and of half-lives of viral RNA and proteins.

The 3' LTR provides signals for viral replication in addition to those for transcriptional termination. Thus, to minimize helper virus production, it will be desirable to replace the 3' LTR with a heterologous poly(A) signal sequence. Also, it will be desirable to include a drug-resistance marker gene for cell selection. Thus, the 3' LTR of selected clones was substituted with a puromycin-poly(A) cassette. Analysis showed that both the wild-type and double deletion mutant could tolerate this substitution without a marked adverse effect on RNA expression relative to the parental unsubstituted clone. Similarly, the insertion of the puromycin gene at the *nef* site of the clone with an intact 3' LTR was not detrimental for RNA expression.

Packaging: The effect of deletions and substitutions of the provirus on RNA encapsidation is shown in Fig. 2. This figure contains the actual data for relative levels of viral RNA and core antigen in viral particles, and these data have not been normalized with respect to either the level of the intracellular viral RNA or the extracellular viral particles. Normalization requires a number of assumptions, including one that the half-life or turnover rate of RNA, which occurs inside the cell but not in viral particles, is not affected by the deletions and substitutions. Normalization with respect to the intracellular RNA also does not distinguish between RNA species destined for encapsidation from those destined for translation, and assumes that there is no effect of genetic manipulation on these destinies.

Evidently, the smaller deletion downstream of the splice donor site (clone PK2) did not affect viral particle production nor did it significantly affect RNA encapsidation. The larger deletion in this region (clone PK36) reduced RNA encapsidation without significantly affecting viral particle production. The deletion of the upstream region (clone SK36) seems to have a slightly greater effect on RNA encapsidation than the downstream deletion (clone PK36). A deletion encompassing the splice donor site (clone PK8) reduced both viral particle production and viral RNA encapsidation. This was expected because this clone did not generate appreciable steady-state levels of vector RNA inside the cell (Fig. 1). The clone with deletion of the leader sequence region both upstream and downstream (clone SD36) displayed lowered viral particle production, but this reduction was only 30–50% of the wild type. In contrast, this clone was severely attenuated in its ability to encapsidate viral RNA.

Replacement of the 3' LTR of this clone with the puromycin-

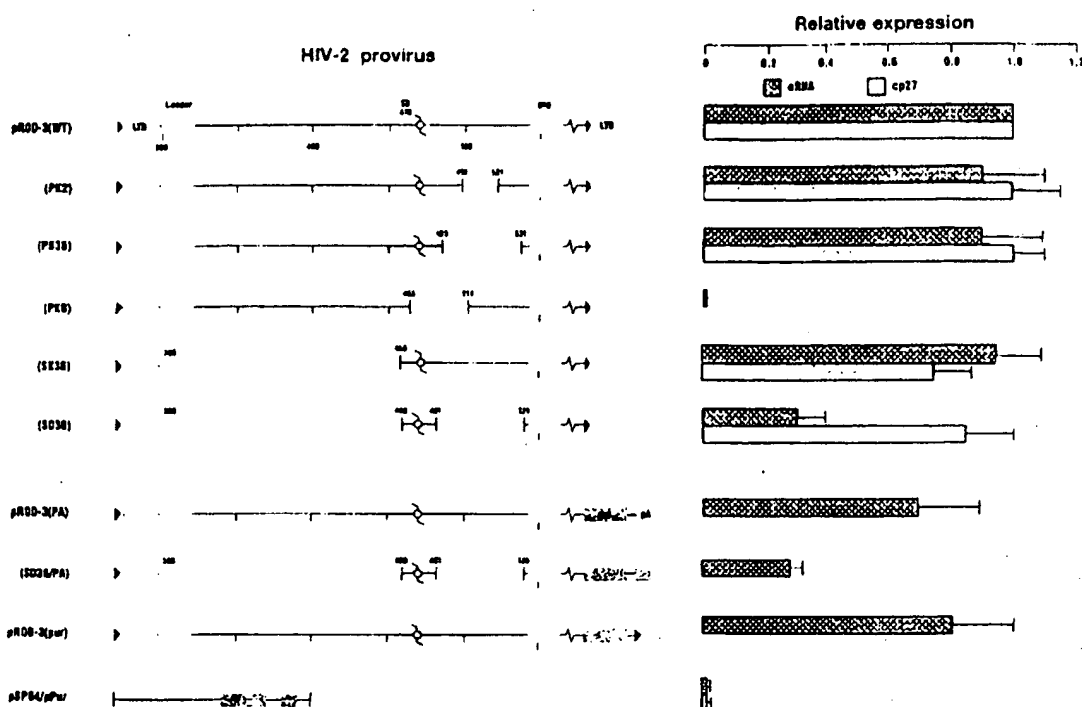


FIG. 1. Effect of leader sequence deletions on the expression of HIV-2(ROD) in human epitheloid 293 cells. About $0.5\text{--}1.0 \times 10^6$ cells were transfected with $8\text{--}12 \mu\text{g}$ of DNA by the calcium phosphate protocol. Three days later, aliquots of cells were processed to obtain cellular extracts and assayed for the content of HIV-2 p27 by antigen capture assays. Aliquots of cells were also processed to isolate RNA by the RNAzol extraction procedure as described in Material and Methods, which includes DNase digestion. Samples of RNA were slot-blotted on to a nitrocellulose membrane and hybridized with ^{32}P -labeled HIV-2-specific probe. Hybridization signals reflecting viral RNA abundance were quantitated with the aid of a Phosphor-Image analyzer (Molecular Dynamics, Sunnyvale, CA). The intracellular levels of HIV-2 p27 antigen and RNA synthesized by mutant clones are expressed relative to the wild-type clone. The p27 antigen level observed for the wild-type clone ranged from 30 to 50 ng/ml, corresponding to about $0.1\text{--}0.5 \times 10^6$ cells. In some cases, the relative abundance of intracellular viral RNA was further checked by Northern blot hybridization and the results were similar to those obtained by slot-blot hybridization.

poly(A) cassette did not further change its phenotype, it continued to produce appreciable levels of viral particles that were deficient in viral RNA. Replacement of the 3' LTR in the wild-type clone with the puromycin poly(A) cassette (clone PA) resulted in about 50% reduction in the observed viral RNA encapsidation. A smaller degree of reduction in RNA encapsidation was also observed for the insertion of puromycin gene alone in the wild-type provirus (clone PUR).

Helper Virus Production: To evaluate the presence of replication-competent viral particles in supernatants of transfected cultures, the supernatants were used to infect CD4^+ CEM cells as targets and cultures monitored visually for syncytia formation and for progeny p27 core production (Table 1). The supernatants from cultures transfected with clones PK2 and PK36 contained replication-competent, syncytia-inducing viral particles approaching the levels of cultures transfected with the wild-type clone. The supernatants from clone SK36-transfected cultures were also positive for syncytia induction. Thus, neither upstream nor downstream deletion alone resulted in a helper virus-free phenotype. In contrast, supernatants from cultures

transfected with clone SD36 were essentially negative for replication-competent viral particles. However, the cultures secondarily infected with this supernatant sometimes presented evidence for visually observable but minimal syncytia formation. The phenotype of the particles contained in the supernatant of clone SD36/PA-transfected cultures was similar to those from clone SD36 transfected cultures with rare, if any, syncytia induction. Although not extensively investigated, the insertion of puromycin gene into the wild-type (clone PUR) appeared to attenuate its ability to produce infectious transmissible viral particles. Although the RNA encapsidation phenotype of the clone PUR was about 70% of the wild-type clone (Fig. 2), the transmissible infectivity of the particles produced by this clone appeared to be about 20% of the wild type.

Human lymphoid CEM cells

Expression: The viral RNA expression and syncytia induction phenotype of mutant clones PK2 and PK36 with deletions downstream of the splice donor site were only modestly dif-

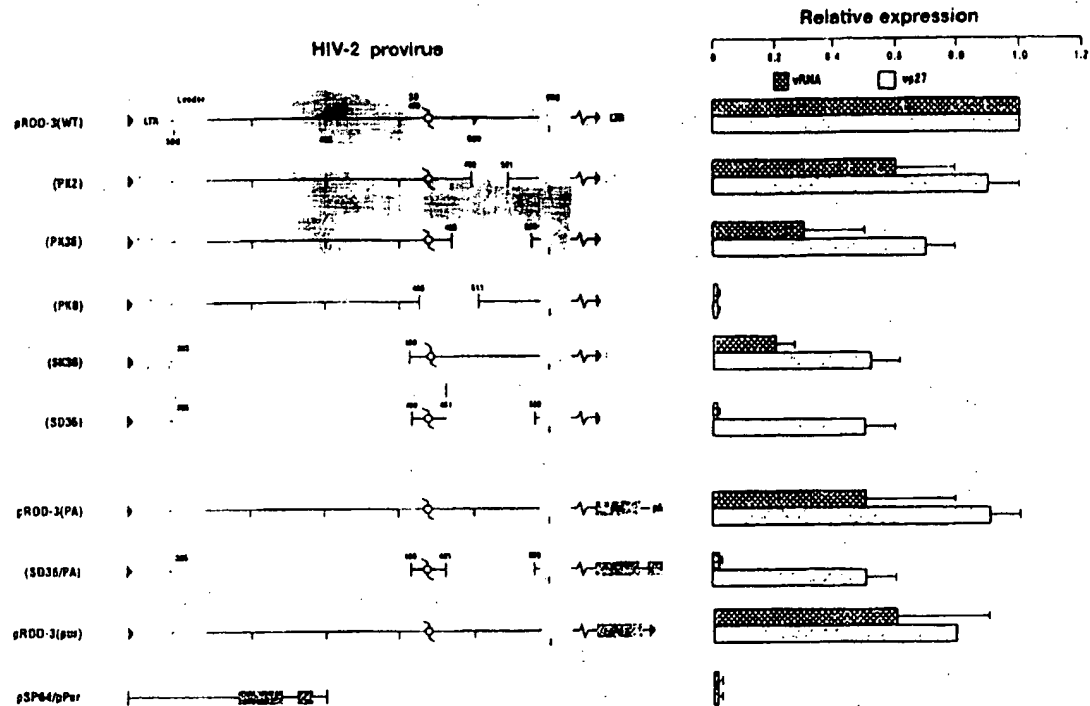


FIG. 2. Effect of leader sequence deletions on the packaging of viral RNA and proteins HIV-2(ROD) in human lymphoid 293 cells. Cells were transfected with the DNA as described in the legend to Fig. 1. Culture supernatants were collected, an aliquot was used for p27 antigen determination, and the remaining supernatant was used for isolation of particle-associated viral RNA. Virus particles in the supernatant were collected by centrifugation through a column of glycerol as described in Materials and Methods. The pellets were lysed with RNazole and RNA purified as described above, including digestion with DNase. RNA was slot-blotted, hybridized with 32 P-labeled HIV-2-specific probe and quantitated as described in the legend to Fig. 1. The data for mutant clones are expressed relative to the wild-type clone. The level of supernatant p27 antigen ranged from 10 to 50 ng/ml. The amount of particle-associated RNA relative to that present in the cell for the wild-type clone was roughly estimated to be 10% for the wild type.

ferent from that of the wild-type clone (Fig. 3). In some experiments, these mutant clones synthesized viral RNA, which exceeded by 20–40% the level of viral RNA synthesized by the wild-type clone. Clone PK8 with deletion of the splice donor site was inactive both transcriptionally and in syncytia induction. Clone SD36 with deletions both upstream and downstream of the splice donor site was severely attenuated in viral RNA synthesis but appeared not to be as severely attenuated in syncytium induction. The fact that this clone induced observable syncytia despite reduction in viral RNA abundance suggests that syncytium induction is not directly proportional to viral RNA synthesis. Substitution of the 3' LTR with the puromycin-poly(A) cassette may have further attenuated the phenotype of this clone because clone SD36/PA was even less effective in syncytia induction than clone SD36. Insertion of the puromycin gene in the 3' region at the *nef* site was also detrimental to the ability of the clone to synthesize or accumulate viral RNA and induce syncytia formation.

Packaging: Although the deletion of the downstream region (clones PK2 and PK36) did not remarkably affect viral particle production, it had a noticeable effect on viral RNA packaging (Fig. 4, Table 2). For example, clone PK36 produced particles

whose overall viral RNA content was about one-third of those produced by the wild-type provirus. These results suggest that the mutant was producing more empty or RNA-deficient particles than the wild-type clone. As expected, the mutant clone with the deletion of the splice donor site (clone PK8) neither pro-

TABLE 1. INFECTIVITY IN HUMAN LYMPHOID CEM CELLS OF PROGENY VIRUS PRODUCED BY TRANSFECTED HUMAN EPITHELOID 293 CELLS

Clone	Syncytia induction	Progeny p27
pROD-3 (WT)	+++	1.0
(PK2)	+++	1.0 \pm 0.0
(PK36)	+++	0.6 \pm 0.2
(PK8)	(-)	\leq 0.01
(SK36)	+	0.15 \pm 0.05
(SD36)	\pm	\leq 0.01
pROD-3(PA)	+	0.03 \pm 0.02
(SD36/PA)	\pm	\leq 0.01
pROD-3(PUR)	++	0.2
pSP64/pPur	(-)	(-)

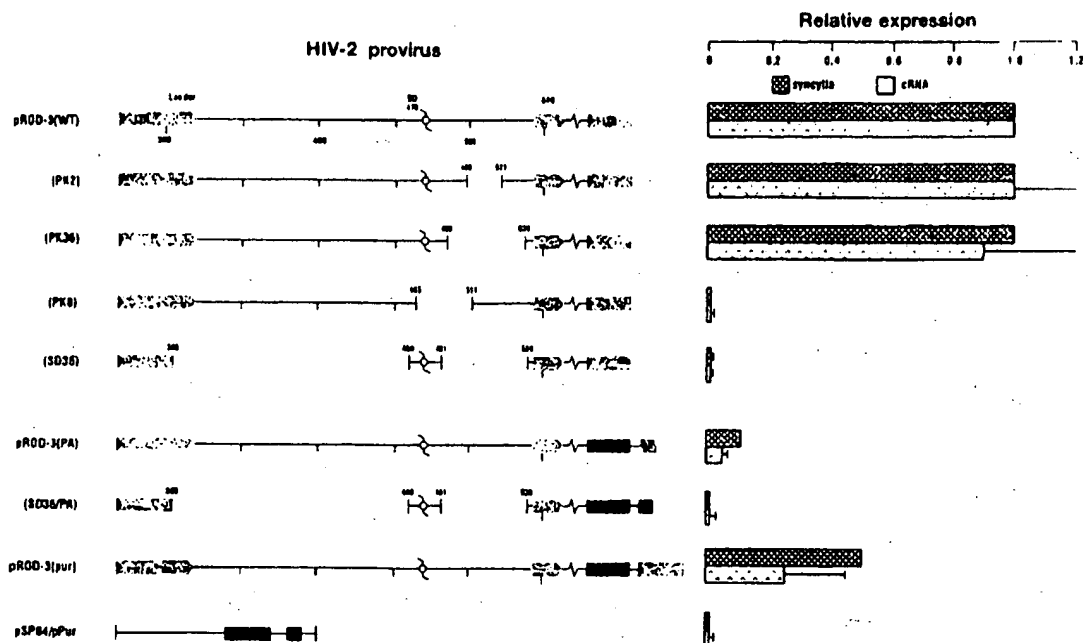


FIG. 3. Effect of leader sequence deletions on the expression of HIV-2(ROD) in human lymphoid CEM cells. About $4-8 \times 10^6$ cells were transfected with $4-8 \mu\text{g}$ of DNA by the DEAE-dextran protocol. Five days later, cultures were examined for the presence of syncytia and these were visually estimated in cultures transfected with mutant relative to those in cultures transfected with the wild-type clone. Because the syncytia were not actually counted, these estimates are approximate. Cells were harvested and processed for RNA isolation by the RNazole procedure, including DNase digestion, and the abundance of virus-specific RNA was determined by slot-blot hybridization as described in the legend to Fig. 1.

duced particles nor encapsidated viral RNA. Mutant clone SD36, with deletions upstream and downstream of the splice donor site, also did not produce viral particles or encapsidate viral RNA. Replacement of the 3' LTR with puromycin-poly(A) cassette (clone PA) had a marked detrimental effect on viral production and viral RNA encapsidation. Although this clone produced a detectable level of viral particles, their RNA content was too low to be measured reliably by the assay used, which was not based on the PCR amplification of the RNA. The clone with the upstream and downstream deletion as well as replacement of the 3' LTR with the puromycin-poly(A) cassette (clone SD36/PA) was essentially negative in its ability to produce viral particles or RNA. The insertion of the puromycin gene in the 3' region of the wild-type clone at the *nef* site (clone PUR) appeared to attenuate viral particle production. The phenotype of this provirus in CEM cells was not investigated extensively.

Structure of HIV-1 and HIV-2 leader sequence

Figure 5 shows the predicted secondary structure of the HIV-2(ROD) leader sequence, which displays a number of stem-loops. Depending on the definition, there may be six to eight such stem-loops compared with four stem-loops reported for the HIV-1 leader sequence (Clever and Parslow, 1997; McBride *et al.*, 1997). Furthermore, the leader sequence of

HIV-2 is longer (547 nucleotides) than the corresponding sequence of HIV-1 (343 nucleotides) and there is only a marginal sequence homology between them.

DISCUSSION

Gene transfer for disease treatment or trait enhancement is becoming increasingly realistic. The ability to treat not only monogenic but also multigenic diseases may not be very far away. To avoid bottlenecks, concurrent progress in developing gene transfer vectors is required. For long-term gene transfer, retroviral vectors remain the vectors of choice; however, currently used murine retroviral vectors suffer from a number of shortcomings for human gene therapy. These include the inability to transduce nondividing cells or to achieve regulated expression of the transgene. Also, they are subject to human complement-mediated inactivation. Novel approaches to address these issues though are being explored (Cosset *et al.*, 1995; Hwang and Anderson, 1997). Human lenti-retroviruses, including HIV-1 and HIV-2, have many desirable features. These include their ability to transduce nondividing cells and allow the achievement of regulated expression as a result of their possession of a number of regulatory loops. We think HIV-2 may be even better than HIV-1 in this regard. HIV-2 as a group is

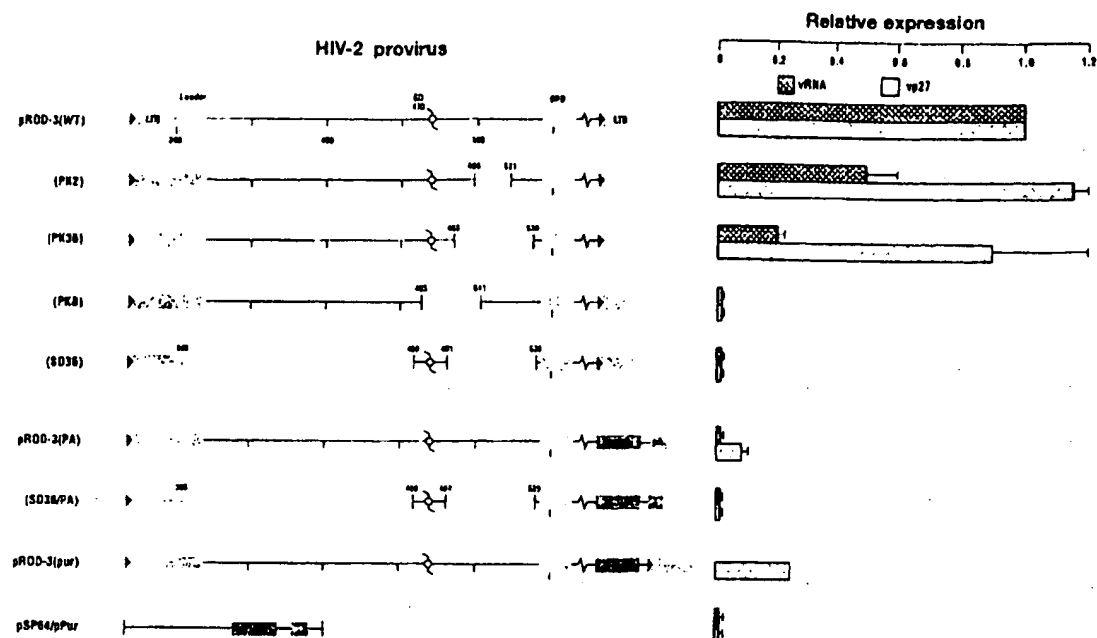


FIG. 4. Effect of leader sequence deletion on the packaging of viral RNA and proteins of HIV-2(ROD) in human lymphoid CEM cells. Cells were transfected and extracellular particle-associated viral RNA and p27 antigen were analyzed as described in the legend to Fig. 3. The results for mutant clones are expressed relative to the wild-type clone. The level of the supernatant p27 for the wild-type clone was about 10–40 ng/ml. The estimated level of packaging of viral RNA relative to the intracellular viral RNA for the wild-type clone was about 10%, a level similar to that observed with 293 cells.

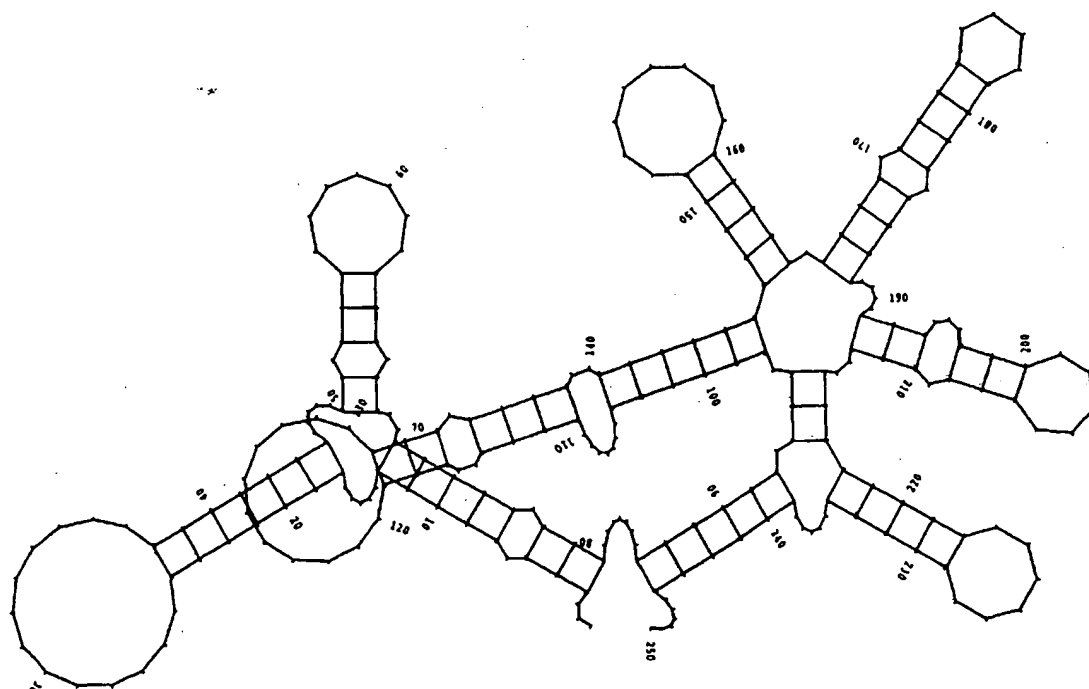


FIG. 5. Predicted secondary structure of the leader sequence of HIV-2(ROD) showing multiple stem-loops.

less pathogenic than HIV-1; thus, it is safer to manipulate during design and production stages. The genes imparting the ability to transduce nondividing cells are more suitably configured in HIV-2 than in HIV-1. The desirable nuclear import function in HIV-1 is mainly encoded by the R gene, which also has undesirable cell cycle arrest function (Connor *et al.*, 1995; Rogel *et al.*, 1995; DiMarzio *et al.*, 1995; Mahalingam *et al.*, 1997). These two functions—nuclear import and cell cycle arrest—are segregated in HIV-2, being encoded by X and R genes, respectively (Fletcher *et al.*, 1996). Because of the limited relatedness between HIV-1 and HIV-2 at the genetic level (Kumar *et al.*, 1990), the HIV-2 vector is less likely to recombine with the resident HIV-1 genome in HIV-1 infection. This will minimize the risk of any enhanced pathogenicity of the recombinant in the individual and of transmission to the bystanders. Moreover, HIV-2 itself may downregulate HIV-1 expression (Arya and Gallo, 1996b; Al-Harhi *et al.*, 1998).

A central requirement in developing lentiviral vector systems is the knowledge of the packaging signal, a sequence element to be included in the transfer vector to achieve encapsidation of transgene and excluded from the packaging vector to avoid helper virus production. In a previous study analyzing the leader sequence-based packaging signals of HIV-2, we noted that the sequence element downstream of the splice donor site and upstream of the *gag* ATG contributed to, but was not the sole determinants of, packaging (Garzino *et al.*, 1995). A later study amended this observation and may have inadvertently created the impression that we had concluded that the downstream elements were necessary and sufficient for HIV-2 packaging (McCann and Lever, 1997).

A specific deletion within the downstream elements, but at a distance from the splice donor site, did not significantly affect the expression of viral RNA or proteins either in human epithelioid or lymphoid cells. However, these downstream deletions did affect packaging; the magnitude of effect depended on the extent of deletion, with the larger deletion causing a 40–80% defect in encapsidation relative to the wild type. Nonetheless, these mutant proviruses with the downstream deletions continued to produce unacceptable level of infectious viral particles. The fact that CEM cultures transfected with these mutants had a lower helper virus titer than transfected 293 cultures may be related to the amplification of the primary defect by further transmission in CD4⁺ CEM but not in the CD4⁺ 293

cells. Similarly, a deletion located exclusively upstream of the splice donor site had detrimental effect on encapsidation (60–80% reduction) but was not helper virus free in its phenotype. Thus, sequence elements located both upstream and downstream of the splice donor site in the leader sequence contribute to RNA encapsidation, and neither one can be ignored in designing helper virus-free packaging vectors and transfer vectors.

The effect of the combined upstream and downstream deletion on expression was more marked and appeared to depend on the cell type. In 293 cells, this deletion did not significantly affect level of viral proteins, but caused readily observed reduction in the steady-state level of viral RNA. The reason for the discrepancy is not clear. It may be related to the differences in half-lives of viral RNAs encoded by the mutant as compared with those encoded by the wild-type provirus. For example, it is possible that the deletion negatively impacts the turnover rate of viral RNA without affecting the turnover rate for viral proteins. The upstream sequence is located in the 5' untranslated region of viral RNAs and is not normally spliced out. The deletion of this sequence could augment the export and translation of viral RNA. The combination of the negative effect on turnover and positive effect on utilization may result in lower steady-state level of viral RNA and higher level of viral proteins for the mutant as compared to the wild-type provirus.

The combined deletion had the desired effect on the packaging. Although the deletion was accompanied by some loss of viral particle production (up to 50% relative to the wild type), the particle thus produced contained little, if any, viral RNA. Thus, this mutant provirus produced RNA deficient helper viral particles with little or no infectivity. However, the lack of infectivity was not complete because occasionally low levels of syncytia were observed in secondary target cultures. In contrast to 293 cells, the combined deletion was accompanied by a severe apparent attenuation of expression and consequently also of packaging in CEM cells. One interpretation of this difference between 293 and CEM cells is that the readout for CEM cells is a consequence of not only of the primary expression and packaging, but also included the amplification of the primary event. There could also be some direct cell-type-dependent effect of the sequence elements.

Notably, deletion of the splice donor site itself resulted in the dramatic reduction in the expression of viral RNA, independent of the cell type. We do not know yet if that was because of the reduction in the rate of viral RNA synthesis or its export and acceleration of its degradation. The observation is consistent with the idea that the RNA species destined for splicing, if not spliced, are degraded and not just restricted to the nuclear compartment (Schwartz *et al.*, 1992; Malim and Cullen, 1993).

The multipartite nature of the leader sequence-based packaging signal of the HIV-1 leader sequence was recently reported (McBride *et al.*, 1997). The leader of HIV-1 contains multiple stem-loop structures termed SL1–SL4 and, in the kissing loop model, these structures participate in RNA dimerization and packaging (Berkhout and Van Wamel, 1996; Paillart *et al.*, 1996; Laughrea *et al.*, 1997; Clever and Parslow, 1997). The primary sequence of the leader of HIV-2 shows only marginal homology with that of HIV-1 and it is much longer in HIV-2 than in HIV-1. The predicted secondary structure of the HIV-

TABLE 2. INFECTIVITY IN HUMAN LYMPHOID CEM CELLS OF PROGENY VIRUS PRODUCED BY TRANSFECTED CEM CELLS

Clone	Syncytia induction	Progeny p27
pROD-3 (WT)	+++	1.0
(PK2)	+++	0.4 ± 0.2
(PK36)	+++	0.2 ± 0.1
(PK8)	(-)	≤0.01
(SD36)	(-)	0.03
pROD-3(PA)	(-)	0.07
(SD36/PA)	(-)	≤0.01
pROD-3(PUR)	ND*	ND
pSP64/pPur	(-)	(-)

*ND, Not determined.

2 leader RNA also presents a number of stem-loop structures, but it is more complex and displays six to eight stem loops, depending on their definition. We think it is worth emphasizing that the primary as well as secondary structure of the HIV-2 leader is quite unlike that of HIV-1 leader. Whether the two viruses follow a similar pathway for dimerization and packaging is yet to be determined.

To ensure that a packaging vector could be designed that was helper virus free but maintained the capacity to express the viral gene needed for *in trans* packaging, the requirement of the 3' LTR for second-strand synthesis, its reverse transcription, and viral transmission was exploited. Thus, the double-deletion mutant provirus was further modified by the replacement of its 3' LTR with a heterologous transcriptional termination signal. This time, a puromycin resistance gene for eventual drug selection of the transduced cells was also included. The hope was that this modification would not affect the expression capability of the vector but would further curtail helper virus production. This hope was fulfilled, as the replacement of the 3' LTR by the puromycin-poly(A) cassette in the double mutant did not adversely affect its expression. Surprisingly, the replacement of the 3' LTR with the puromycin-poly(A) cassette in wild-type provirus was accompanied by noticeable decline in viral RNA encapsidation. Because this decline appeared to be due more to the placement of a foreign gene in the 3' region than to the replacement itself, it implies that the 3' region of the viral RNA may influence RNA encapsidation in HIV-2. We have speculated that for HIV-2 viral RNA dimerization may be important for packaging and that the dimerization involves sequence elements dispersed in the viral genome and not just confined to the leader sequence. We envision that the sequence elements, in addition to the leader, induce the formation of initial pool of metastable structures from which more stable dimeric structures are chosen by the components of the packaging machinery.

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